Serotonin-Induced Growth of Pulmonary Artery Smooth Muscle Requires Activation of Phosphatidylinositol 3-Kinase/Serine-Threonine Protein Kinase B/Mammalian Target of Rapamycin/p70 Ribosomal S6 Kinase 1

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We have previously found that both mitogen-activated protein kinase (MAPK)- and Rho kinase (ROCK)-related signaling pathways are necessary for the induction of pulmonary artery smooth muscle cell (SMC) proliferation by serotonin (5-hydroxytryptamine [5-HT]). In the present study, we investigated the possible additional participation of a phosphatidylinositol 3-kinase (PI3K)/serine-threonine protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/p70 ribosomal S6 kinase (S6K1) pathway in this growth response. We found transient activation of Akt (Ser^{473}) and more prolonged activation of S6K1 by 5-HT. Inhibition of PI3K with Wortmannin and LY294002 completely blocked these activations, but not that of MAPK or the ROCK substrate myosin phosphatase targeting subunit. Similarly, inhibition of MAPK and ROCK failed to block the Akt activation. Inhibition of Akt with NL-71–101 and downregulation of Akt expression with Akt small interfering RNA blocked 5-HT–induced S6K1 phosphorylation. Wortmannin, LY294002, and NL-71–101 dose-dependently inhibited 5-HT–induced SMC proliferation. 5-HT stimulated mTOR phosphorylation and the mTOR inhibitor, rapamycin, blocked activations of S6K1 and S6 ribosomal protein, and inhibited 5-HT–induced SMC proliferation. Akt phosphorylation and cell proliferation were also blocked by the antioxidants, N-acetyl-l-cysteine, *Ginko biloba* 501, and tiron, the reduced nicotinamide adenine dinucleotide phosphate oxidase inhibitor, diphenyleneiodonium, and the 5-HT2 receptor antagonists ketanserin and mianserin, but not by the 5-HT serotonin transporter or 5-HT 1B/1D receptor antagonists. We conclude from these studies that a parallel PI3K- and reactive oxygen species–dependent Akt/mTOR/S6K1 pathway participates independently from MAPK and Rho/ROCK in the mitogenic effect of 5-HT on pulmonary artery SMCs. From these and other studies, we postulate that independent signaling pathways leading to 5-HT–induced SMC proliferation are initiated through multiple 5-HT receptors and serotonin transporter at the cell surface.

Keywords: mammalian target of rapamycin; p70 ribosomal S6 kinase; phosphatidylinositol 3-kinase; pulmonary hypertension; serine/threonine protein kinase; serotonin; smooth muscle cells

We have previously demonstrated that extracellular signal-regulated kinase (ERK) 1/ERK2 mitogen-activated protein kinase (MAPK) (1, 2) and Rho/Rho kinase (ROCK) (3) play important roles in 5-hydroxytryptamine (5-HT)–induced smooth muscle cell (SMC) proliferation, and have concluded from our studies that these pathways operate in parallel in their proliferative effects. Although ERK1/ERK2 MAPK activation by 5-HT occurs independently from that of Rho/ROCK, the concomitant activation of Rho/ROCK is important to facilitate translocation of the activated ERK into the nucleus. In turn, ERK MAPK activates transcription factors in the nucleus that participate in cell cycling.

Another signaling pathway that is known to participate in cellular proliferation is the phosphatidylinositol 3-kinase (PI3K)–mediated serine-threonine protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/p70 ribosomal S6 kinase 1 (S6K1) pathway (4, 5). The PI3K/Akt signal transduction cascade has been investigated extensively for its role in antiapoptosis and cell survival. This pathway has also been associated with regulation of cell growth and cycle progression (6–9). In response to a wide variety of stimuli, including growth factors and cytokines, PI3K phosphorylates the lipid phosphatidylinositol-4,5-biphosphate at the 3′-OH position to generate the lipid second messenger phosphatidylinositol-3,4,5-triphosphate. Phosphatidylinositol-3,4,5-triphosphate then recruits its downstream effector, Akt, to the plasma membrane, where it is phosphorylated and activated. Studies have shown that activated Akt directly phosphorylates tuberous sclerosis complex (TSC) 2, leading to the inhibition of function of the TSC1/TSC2 complex (10, 11). In turn, a small GTPase, Rheb, is inactivated by TSC1/2 and positively modulates mTOR function (12, 13). In mammals, mTOR phosphorylates the S6K1 and the eukaryotic translation initiation factor 4E binding proteins, resulting in activation of S6K1 and inactivation of 4E binding protein 1. Both mTOR effectors independently regulate protein translation processes to promote cell growth and cell cycle progression (14).

Several G-protein–coupled agonists, including angiotensin II and endothelin 1, have been shown to activate PI3K/mTOR pathways in SMCs (15, 16). However, except in limited studies with neuronal cells (17), this pathway has not been explored in relation to 5-HT–induced cellular responses. In the case of the neuronal cells, the 5-HT 1B receptor was coupled to pathway activation, and cellular proliferation as an end product was not investigated. Because of the paucity of data in this area for pulmonary vascular SMCs, where stimulation by 5-HT may result in SMC proliferation and pulmonary hypertension (18, 19), we undertook the present study to assess possible participation of the PI3K/Akt signaling pathway and downstream effectors S6K1 and S6 ribosomal protein in SMC proliferation produced by 5-HT. Our results show that Akt is activated transiently through PI3K by 5-HT, resulting in a more sustained activation of S6K1 and S6 ribosomal protein. Furthermore, inhibitory studies indicate that this pathway operates in parallel to and independently from those of MAPK and Rho/ROCK, demonstrating

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an additional cell signaling pathway that is required for SMC proliferation produced by 5-HT. Of note, the activation of the PI3K/Akt signaling pathway by 5-HT is dependent upon generation of reactive oxygen species (ROS), and appears to be specifically related to ligation of a 5-HT 2A receptor. In extension of previous data, where SMC proliferation was found to be dependent upon both serotonin transporter (SERT) and 5-HT 1B/1D receptor (3, 20), the dependence of this action on ligation of a 5-HT 2A receptor suggests the novel observation of combined action of 5-HT on both SERT and multiple 5-HT receptors, leading to cellular proliferation.

MATERIAL AND METHODS

Reagents

RPMI 1640 medium was purchased from GIBCO Laboratories (Grand Island, NY). FBS, 5-HT, citalopram, imipramine, GR55562, GR127935, ketanserin, mianserin, SB215505, and 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane were purchased from Sigma Chemical Co. (St. Louis, MO). Y27632 and NL-71–101 were from Calbiochem Inc. (La Jolla, CA). RS102221 was purchased from Tocris Cookson Ltd. (Avonmouth Bristol, UK). Rapamycin, Wortmannin and LY294002, phospho-specific p42/44 MAP kinase (Thr^{202}/Tyr^{204}) antibody, phospho-Akt (Ser^{473}) antibody, Akt antibody, phospho-S6K1 (Thr^{421}/Ser^{424}) antibody, S6K1 antibody, phospho-mTOR (Ser^{2481}) antibody, total mTOR antibody, and phospho-S6 ribosomal protein (Ser^{235}/Ser^{236}) antibody were from Cell Signaling Technology (Beverly, MA). Anti–phospho–myosin phosphatase targeting subunit (MYPT1 [Thr^{696}]) rabbit polyclonal antibody was purchased from Upstate Group, Inc. (Lake Placid, NY). Anti–MYPT-1 and anti-ERK rabbit polyclonal antibodies were from Santa Cruz Biotechnolgy (San Diego, CA). [Methyl-^{3}H] thymidine (1 mCi/ml; specific activity, 6.7 Ci/mmol) was from New England Nuclear Corp. (Boston, MA). Signal-silenced Akt small interfering RNA (siRNA) kit was from Cell Signaling Technology. Nontargeting siRNA control was purchased from Dharmacon (Lafayette, CO).

Cell Culture

SMCs from bovine pulmonary artery were isolated by a modification of the method of Ross, as previously described (20), and were cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin, and 0.5% streptomycin. Cells from passages 3–10 were used in our study.

Incorporation of [^{3}H] Thymidine

SMCs seeded in 96-well plates were growth-arrested for 72 h in medium containing 0.1% FBS. Cells were incubated with and without 1 μmol/liter 5-HT in the same medium for 20 h, then labeled with [methyl-^{3}H] thymidine (20 μCi/ml) for 4 h. In some experiments, inhibitors were added 30 min before the 5-HT. DMSO (0.1%) was added to the vehicle control group. After labeling, experiments were terminated by aspiration of medium, and the cells were harvested onto 96-well microplate filter paper using a Tomtec harvester (Tomtec, Hamden, CT). Radioactivity was countered in a Trilux liquid scintillation and luminescence counter (Perkin Elmer Life Science, Boston, MA).

Preparation of Whole-Cell Lysates

Treated SMCs were rinsed with ice-cold PBS, and then incubated for 15 min at 4°C in radioimmunoprecipitation assay (RIPA) lysis buffer. Lysates were centrifuged at 14,000 × *g* for 10 min to collect supernatants.

siRNA Transfection

siRNA was used to specifically silence Akt in SMCs. The prevalidated single-sequence siRNA duplex was purchased from Cell Signaling Technology. The siRNA was transfected into the cells according to the kit manufacturer. First, cells were plated in 35-mm dishes in medium containing 10% serum. When the cells became 50% confluent, medium was removed from the cells and replaced with 1 ml fresh serum-containing medium. The transfection reagent (4 μl) was diluted with 200 μl serum-free medium and incubated at room temperature for 5 min. Twelve microliters siRNA stock (10 μmol/liter) was added to the 200 μl diluted transfection reagent to yield a concentration of 100 nmol/liter. The solution was mixed and incubated for 5 min at room temperature. Then, 200 μl of the siRNA and tranfection reagent complexes were added to the dishes containing 1 ml medium. Control cells were treated with the same amount of transfection reagents, and the nonspecific siRNA control cells were transfected with 100 nmol/liter nontargeted siRNA control. After 24 h of transfection, the medium was replaced with fresh serum-free medium, and this was incubated at 37C° in 5% CO_2 for 48 h before performing experiments.

Western Blot Analysis

Phosphorylation of Akt, ERK, MYPT1, S6K1, and S6 were analyzed using phospho-specific rabbit polyclonal antibodies. Immunoreactive bands were bonded with horseradish peroxidase–conjugated secondary antibodies and subsequently visualized using an ECL Chemiluminescent Western Blotting Detection kit (Pierce, Rockford, IL).

Quantification of bands was done by gel densitometry with Sigma gel analysis software, and protein phosphorylation was normalized by total protein-band densitometry individually.

Statistical Analysis

Means ± SD were calculated and statistically significant differences among groups were determined by one-way ANOVA analysis followed by the Tukey's *post hoc* comparisons. An effect was considered significant when $P < 0.05$.

RESULTS

Time Course of Activation of Akt, S6K1, and Ribosomal S6 Protein by 5-HT

For comparison, and consistent with our previous studies, stimulation of pulmonary artery SMCs (PASMCs) with 5-HT (1 μmol/liter) caused both MAPK and ROCK activation, as indicated by transient ERK and MYPT1 protein phosphorylation (Figure 1A). We further examined the effect of 5-HT on phosphorylation of Akt, S6K1, and S6. 5-HT induced a rapid phosphorylation of Akt at Ser^{473}, with a peak in 3–5 min. The activation of mTOR downstream effector S6K1 was measured as the readout of mTOR function. We found that 5-HT caused markedly and prolonged S6K1 phosphorylation at Thr^{421}/Ser^{424}. The S6K1 substrate, S6 ribosomal protein, was also strongly phosphorylated in SMCs by 5-HT. This effect was evident at 10 min, and increased for at least 60 min.

5-HT Activates PI3K and Akt Independently of ERK and ROCK

Because PI3K, Akt, ERK, and ROCK are all activated by 5-HT, we examined whether there is a relationship between PI3K/Akt activation and the activation of ERK and ROCK. Inhibition of PI3K with Wortmannin and LY294002 completely blocked 5-HT–induced Akt phosphorylation, but had no effect on 5-HT–induced ERK- and ROCK-targeted MYPT1 phosphorylation (Figures 2A–2C). Similarly, inhibition of Akt with NL-71–101 did not influence 5-HT–induced MYPT1 (Figure 2D) or ERK (Figure 3B) phosphorylation. Inhibition of ERK and ROCK with U0126 and Y27632, respectively, failed to prevent Akt phosphorylation (Figure 2E). These findings show that PI3K is upstream of Akt, and not of ERK and ROCK. Also, ERK and ROCK are not activated by Akt, and, conversely, Akt is not activated by Erk or ROCK. Thus, 5-HT activates Akt, ERK, and ROCK independently in PASMCs.

Inhibition of PI3K, Akt, or mTOR Blocks S6K1 Phosphorylation by 5-HT

Our data showing that rapamycin inhibits 5-HT–induced cellular proliferation (Figure 4D) indicate that mTOR is a regulator of 5-HT–induced mitogenesis. Western blot analysis showed that 5-HT caused strong and prolonged phosphorylation of S6K1 and S6 (Figure 1), demonstrating that the mTOR/S6K1 pathway is

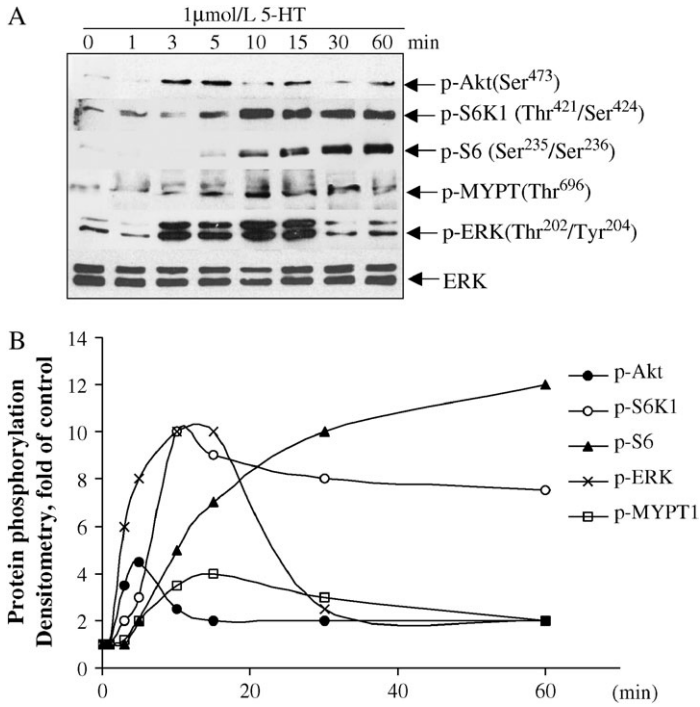

Figure 1. Time course of activation of serine-threonine protein kinase B (Akt), p70 ribosomal S6 kinase (S6K1), and S6 by 5-hydroxytryptamine (5-HT). (*A*) Growth-arrested smooth muscle cells (SMCs) were stimulated with 1 μmol/liter 5-HT for the indicated times. Phosphorylation of Akt (Ser^{473}), S6K (Thr^{421}/Ser^{424}), S6 ribosomal protein (Ser^{235}/Ser^{236}), myosin phosphatase targeting subunit (MYPT1 [Thr^{696}]), and extracellular signal-regulated kinase (ERK [Thr^{202}/Tyr^{204}]) were determined by Western blot analysis using phospho-specific antibodies. The level of total ERK in the whole cell lysis was determined by Western blot analysis using the ERK antibody as a loading control. All experiments were repeated three times and presented as a representative blot. (*B*) The band densitometries were measured with Sigma-gel software and protein phosphorylation was calculated as the relative fold increase in band intensity compared with untreated controls. p-Akt, *filled circles*; p-S6K1, *open circles*; p-S6, *triangles*; p-ERK, *crosses*; p-MYPT1, *squares*.

functionally activated by 5-HT. To further investigate the relationship between PI3K, Akt, and mTOR in regulating 5-HT–induced activation of S6K1, we examined the effect of PI3K, Akt, and mTOR inhibitors on 5-HT–induced S6K1 phosphorylation. Inhibition of PI3K with Wortmannin or LY294002 markedly blocked S6K1 phosphorylation, and rapamycin blocked 5-HT–induced S6K1 phosphorylation (Figures 3A and 3B). The Akt inhibitor NL-71–101 blocked 5-HT–induced S6K1 phosphorylation to a similar degree as did LY94002 (Figure 3B). As shown in Figure 3B, NL-71–101 did not reduce the Akt phosphorylation caused by 5-HT, as this Akt inhibitor competitively inhibits the Akt enzyme catalytic activity, but not the phosphorylation of the enzyme itself (21). To further confirm the specificity of activation of Akt, we examined the action of Akt by using Akt-specific siRNA. Transfection of SMCs with 100 nmol/liter Akt siRNA downregulated cellular Akt expression by ~ 60% without an influence on ERK expression, whereas it markedly reduced phosphorylation of S6K1 by 5-HT (Figure 3C). Neither Akt protein expression nor 5-HT–induced S6K1 phosphorylation was reduced by transfection of cells with the nontargeted control siRNA. These results suggest that PI3K/Akt is upstream of mTOR in mediating the 5-HT–induced S6K1 phosphorylation.

PI3K, Akt, and mTOR Activations Are Required for 5-HT–Induced SMC Proliferation

To test the participation of PI3K, Akt, and mTOR in cellular proliferation caused by 5-HT, cell growth response was measured by [^{3}H]-thymidine incorporation in the presence of pharmacologic inhibitors. Inhibition of PI3K with two structurally unrelated inhibitors, Wortmannin and LY294002, dose-dependently inhibited 5-HT–induced DNA synthesis without significant reduction in basal DNA synthesis (Figures 4A and 4B). To investigate whether Akt is required for this proliferative response, we examined the effect of a selective Akt inhibitor, NL-71–101, which reduced 5-HT–induced [^{3} H]-thymidine incorporation in a dose-dependent manner (Figure 4C). These results indicate that PI3K and its downstream kinase, Akt, are required for 5-HT–induced cell proliferation.

To examine the importance of mTOR in cellular proliferation produced by 5-HT, we tested the effect of the mTOR inhibitor, rapamycin. As shown in Figure 4D, rapamycin inhibited [^{3}H]-thymidine incorporation by 5-HT in a dose-dependent manner. These results indicate that 5-HT–activated PI3K, Akt, and mTOR pathways participate in SMC proliferation.

5-HT Enhances mTOR Protein Phosphorylation

In mammalian cells, the activation of mTOR involves TOR protein phosphorylation. As described previously here, 5-HT markedly stimulated mTOR downstream target S6K1 phosphorylation. To confirm the activation of mTOR by 5-HT in SMCs, mTOR protein phosphorylation was examined. Incubation of cells with 5-HT led to a significant increase in the content of phosphor-mTOR at Ser^{2481} (Figure 5). This suggests that 5-HT induces mTOR/S6K1 activation through modification of mTOR phosphorylation.

Participation of ROS in Akt Phosphorylation by 5-HT

Our previous studies have demonstrated that ROS are required for 5-HT–induced cell proliferation via mediation of ERK activation (1, 2). To investigate whether ROS also participate in 5-HT–induced Akt phosphorylation, we assessed the influence of several antioxidants on this Akt phosphorylation. Structurally unrelated antioxidants N-acetyl-l-cysteine, GK501, and tiron, as well as the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, diphenyleneiodonium, all prevented 5-HT–induced Akt phosphorylation, indicating that 5-HT also regulates Akt phosphorylation via production of ROS (Figure 6).

5-HT 2A Receptors Mediate Akt and S6K1 Phosphorylation

We have previously concluded that 5-HT caused SMC proliferation through combined action of the SERT and the 5-HT 1B/1D receptor (3). From other experiments done in our laboratory using PCR techniques, we have identified that the bovine PASMCs that we study contain 5-HT 1B and 2A receptors,

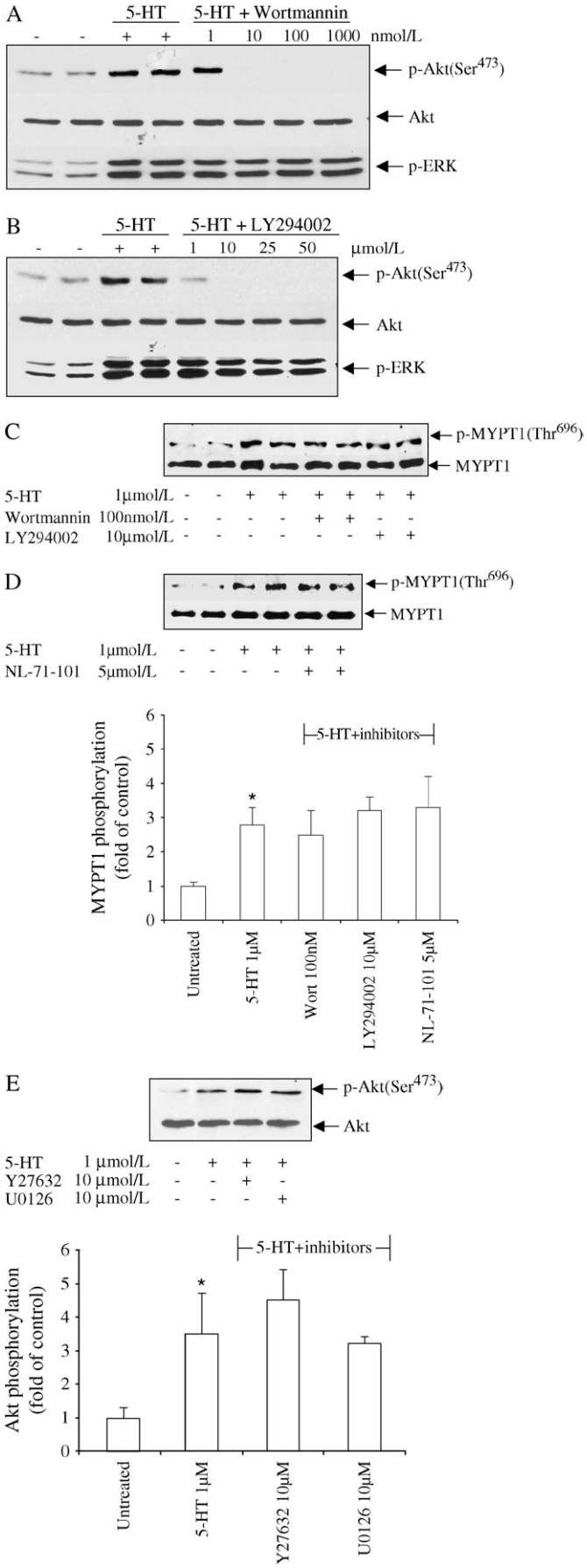

Figure 2. 5-HT activates phosphatidylinositol 3-kinase (PI3K) and Akt independently of ERK and Rho kinase (ROCK). Quiescent SMCs were pretreated with (*A*) 1–1,000 nmol/liter Wortmannin, or (*B*) 1–50 μmol/liter LY294002, for 30 min, then stimulated by 1 μmol/liter 5-HT for 5 min. The phosphorylation of Akt (Ser[473]) and ERK were detected in whole-cell lysates by Western blot analysis using phospho-specific antibody. Total Akt was detected from the same membrane using the total Akt antibody. (*C*) SMCs were pretreated with 100 nmol/liter Wortmannin and 10 μmol/liter LY294002, or (*D*) 5 μmol/liter NL-70–101, for 30 min and then incubated with 1 μmol/liter 5-HT for 15 min. The phosphorylation of MYPT1 (Thr[696]) was detected by Western blot analysis using phospho-specific antibody. (*E*) SMCs were pretreated with 10 μmol/liter Y27632 or 10 μmol/liter U0126 for 30 min and then incubated with 1 μmol/liter 5-HT for 5 min. The phosphorylation of Akt (Ser[473]) was detected by Western blot analysis using phospho-specific antibody. Data in *bar graphs* in (C) and (*D*) represent mean ± SD for $n = 3$. * Significant difference from untreated cells ($P < 0.05$).

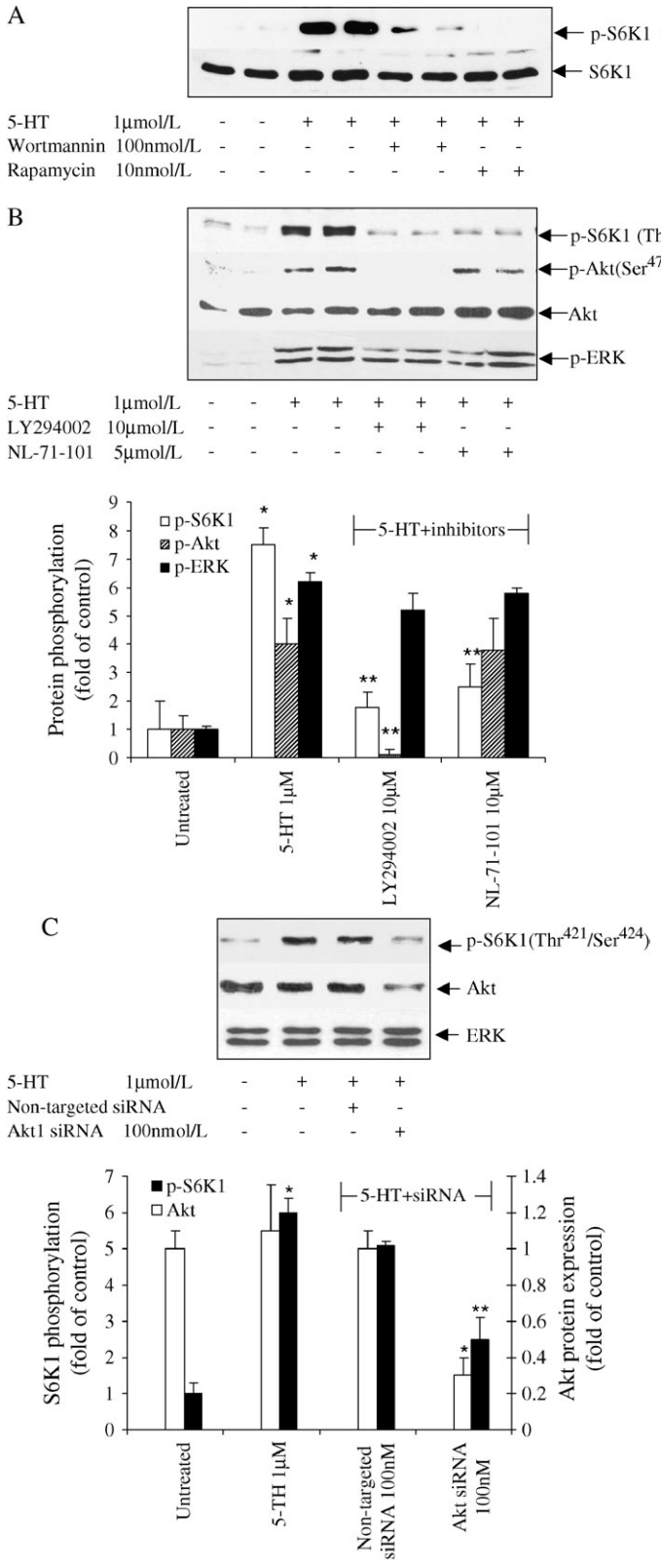

Figure 3. Inhibition of PI3K, Akt, or mammalian target of rapamycin (mTOR) blocks S6K1 phosphorylation by 5-HT. (*A*) Quiescent SMCs were pretreated with 100 nmol/liter Wortmannin or 10 nmol/liter rapamycin for 30 min and then incubated with 1 μmol/liter 5-HT for 10 min. (*B*) Quiescent SMCs were pretreated with 10 μmol/liter LY294002 or 5 μmol/liter NL-70–101 for 30 min and then incubated with 1 μmol/liter 5-HT for 10 min. The phosphorylation of S6K1 (Thr^{421}/Ser^{424}), Akt (Ser^{473}) and ERK (Thr^{202}/Tyr^{204}) were detected by Western blot analysis using phospho-specific antibodies. (*C*) SMCs were transfected with Akt small interfering RNA (siRNA) and nontargeted siRNA (100 nmol/liter) for 24 h. After 48 h starvation in 0.1% FBS medium, cells were treated with 1 μmol/liter 5-HT for 10 min. The total Akt and ERK expressions, as well as the phosphorylation of S6K1 (Thr^{421}/Ser^{424}), were detected by Western blot analysis. The *bar graphs* for blots in (*B*) and (*C*) represent mean ± SD for $n = 3$. * Significant difference from untreated cells ($P < 0$ 0.05); ** Significant difference from 5-HT–treated cells ($P < 0.05$).

along with SERT (R. Day and colleagues, personal communication). To assess if a 5-HT receptor or SERT is required for Akt phosphorylation, we tested the effect of different receptor and transporter antagonists on Akt and S6K1 phosphorylation. Unexpectedly, neither of the SERT inhibitors, imipramine or citalopram (Figure 7A), nor inhibitors of 5-HT 1B/1D receptors (GR55562, GR127935) (Figure 7B), prevented 5-HT–induced Akt phosphorylation. In contrast, inhibition of the 5-HT 2 receptor with ketanserin or mianserin significantly inhibited Akt and S6K1 phosphorylation produced by 5-HT (Figure 7C). Because

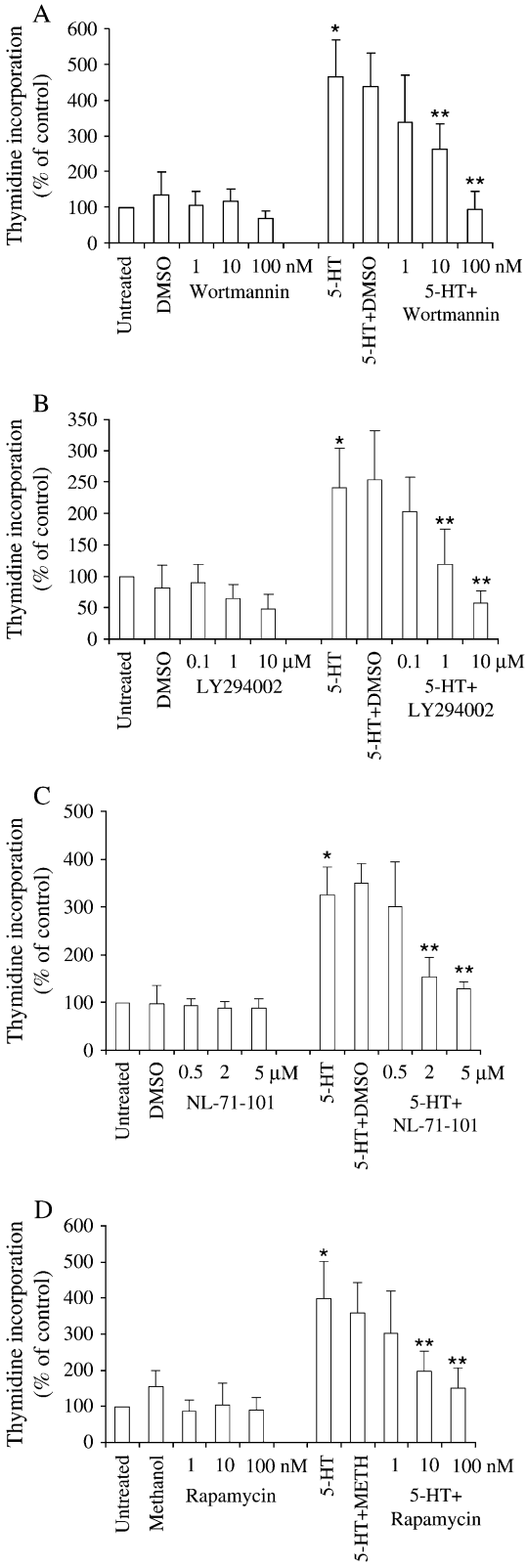

Figure 4. PI3K, Akt, and mTOR activation are required for 5-HT–induced SMC proliferation. Quiescent SMCs were pretreated with PI3K inhibitors Wortmannin (*A*), LY294002 (*B*), Akt inhibitor NL-71–101 (*C*), or mTOR inhibitor rapamycin (*D*) for 30 min. They were then incubated with 1 μmol/liter 5-HT for 24 h. DNA synthesis was determined by monitoring [^{3}H] thymidine incorporation. Data presented are mean ± SD for $n = 3$. *Significant difference from the untreated controls at $P < 0.05$; **significant difference from cells treated with 5-HT alone at $P < 0.05$.

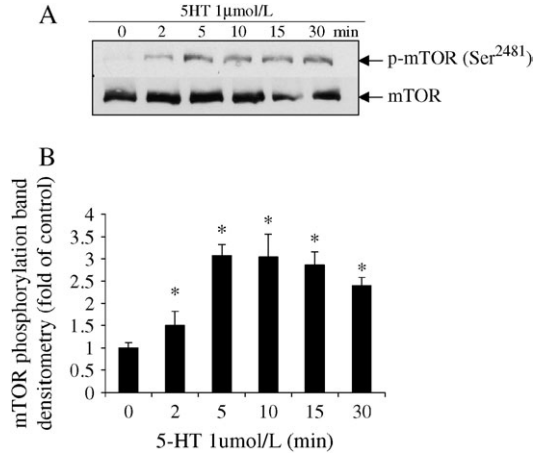

Figure 5. 5-HT upregulates mTOR phosphorylation in bovine pulmonary artery SMCs. (*A*) Quiescent SMCs were treated with 1 μmol/liter 5-HT for the indicated times. The phosphorylation of mTOR (Ser^{2481}) was detected by Western blot analysis using phospho-specific antibody. Total mTOR was detected from the same membrane using the total mTOR antibody. (*B*) Data presented are means ± SD for $n = 3$. *Significant difference from untreated cells at $P < 0.05$.

affinity of the latter agents is relatively low for receptors of the bovine species (22), we tested the effect at 5 μmol/liter concentration. However, inhibition still occurred at concentrations as low as 0.5 μmol/liter (data not shown). The 5-HT 2B receptor antagonist, SB 215505, failed to block these activations (data not shown). The selective 2C inhibitor, RS 102221, showed inhibition only at a concentration of 5 μmol/liter. The 5-HT 2 receptor agonist, 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane, showed a similar, albeit less intense, stimulatory affect on Akt and S6K1 as that of 5-HT (Figure 7D). These results suggest that 5-HT activates PI3K/Akt through a 5-HT 2A receptor, with possible participation of the 5-HT 2C receptor, supporting the concept that a combination of both SERT and various 5-HT receptors are necessary for the 5-HT–induced proliferative effect on SMCs.

DISCUSSION

There is a known association of the 5-HT SERT with hypoxia-induced pulmonary hypertension (23–25), and pulmonary hypertension has been found in patients with platelet storage disease in which serum levels of 5-HT are elevated (26). Furthermore, genetic polymorphism for SERT has been identified in patients with pulmonary hypertension (27, 28). Animal experiments have also identified a relationship between pulmonary hypertension and 5-HT (29–31), and the fawn-hooded rat may be a naturally occurring model of 5-HT–induced pulmonary hypertension (32, 33). Although 5-HT also causes vasospasm of the pulmonary circulation, it has been identified to be a potent stimulant of SMC mitogenesis of the pulmonary vascular wall, leading to chronic pulmonary hypertension (34, 35). There is now considerable interest in the molecular mechanism by which this occurs.

We have found SERT to be an important regulator of SMC proliferation (20), but other investigators have identified 5-HT receptor–related mechanisms for mitogenesis in other cell types (36–38). Furthermore, experimental models of pulmonary hypertension produced by hypoxia have indicated that 5-HT receptors may be involved (39, 40). Our previous studies have provided

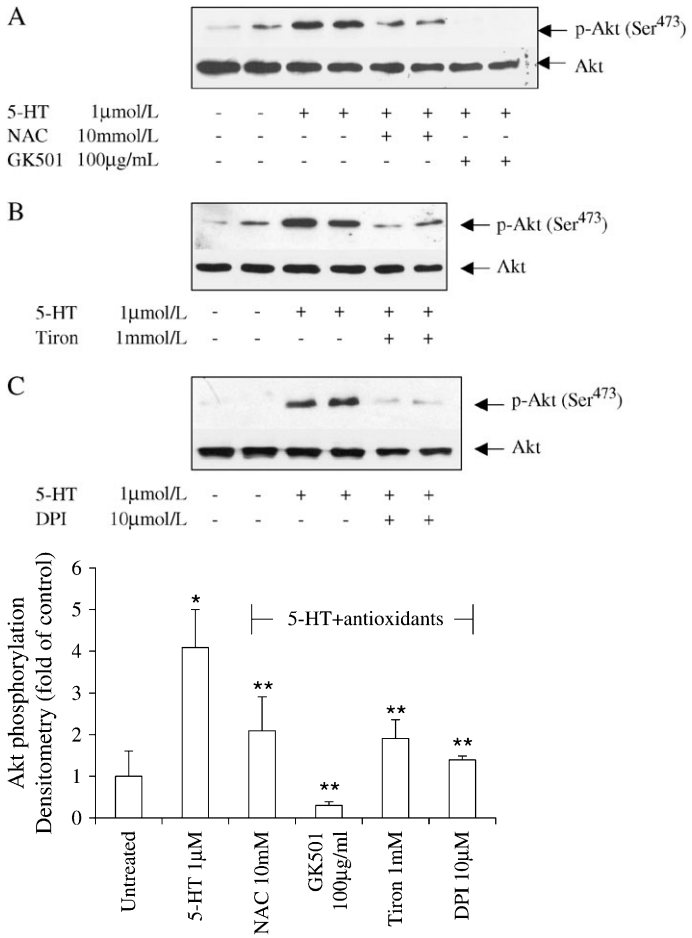

Figure 6. Participation of reactive oxygen species in Akt phosphorylation by 5-HT. (*A*) Quiescent SMCs were pretreated with 10 mmol/liter N-acetyl-l-cysteine (NAC) or 100 μg/ml GK501 for 30 min before treatment with 1 μmol/liter 5-HT for 5 min. (*B* and *C*) SMCs were pretreated with 1 mmol/liter tiron or 10 μmol/liter diphenyleneiodonium (DPI) for 30 min before treatment with 1 μmol/liter 5-HT for 5 min. The phosphorylation of Akt (Ser^{473}) was detected by Western blot analysis using phospho-specific antibody. Total Akt was detected from the same membrane using the total Akt antibody. Data presented are means ± SD for $n = 4$. * Significant difference from untreated cells at $P < 0.05$; ** significant difference from cells treated with 5-HT alone at $P < 0.05$.

evidence that ERK MAPK and Rho/ROCK are intermediate signal transduction pathways leading to SMC proliferation in response to 5-HT in PASMCs (1–3). Their activation is dependent upon the generation of ROS (1, 2). 5-HT activates the ROCK pathway in parallel to that of ERK, and activation of this pathway participates in translocation of ERK into the cellular nucleus (3).

In the present study, we have addressed the possible relationship of PI3K/Akt and mTOR/S6K1 pathways to SMC proliferation produced by 5-HT. Interruption of the pathways blocks 5-HT–induced cellular proliferation. These pathways appear to be in parallel with and unrelated to the ERK MAPK and Rho/ROCK pathways that are also activated by 5-HT (*see* Figure 8). In contrast to ERK MAPK and Rho/ROCK, Akt is activated (at Ser^{473}) via PI3K. The phosphorylation of Akt occurs only transiently, whereas those of S6K1 and S6 are more prolonged. Akt has previously been identified as the downstream target of PI3K in growth-related and survival signaling (9, 41, 42). The response that we observed is similar to a 5-HT–induced increase in Akt activation noted in a neuroblastoma cell line expressing 5-HT 1B receptors (43), but is unlike results reported by Banes and colleagues (44), in which 5-HT failed to activate Akt in rat aortic SMCs. Thus, from these observations, it is apparent that cell signaling involved in the stimulation of SMC mitogenesis produced by 5-HT is much more complex than previously identified, and requires combined activation of ERK MAPK, RhoA/ROCK, and PI3K/Akt pathways.

Our data demonstrate that mTOR is another critical target in mediating 5-HT–induced proliferation in PASMCs, as inhibition of mTOR with rapamycin significantly reduced cellular proliferation caused by 5-HT. S6K1 is a downstream effector of mTOR. Reports have demonstrated activation of S6K1 by 5-HT in Chinese hamster ovary cells and neurons (17, 43). We found that 5-HT also activated S6K1 and its effector, ribosomal S6 protein, in PASMCs. The 5-HT–induced S6K1 phosphorylation at Thr^{421}/Ser^{424} peaked at 10 min, and was sustained for more than 30 min. The phosphorylation of ribosomal S6 protein showed a delayed but similar kinetic profile to that of S6K1. Thus, our data provide the first evidence that 5-HT may stimulate a S6K1-associated translational process in SMCs. We found that both mTOR and PI3K are involved in the regulation of S6K1 activation. Furthermore, using pharmacologic inhibition and specific Akt siRNA, we have demonstrated that Akt is required for S6K1 phosphorylation by 5-HT. These results indicate that mTOR is upstream of S6K1, and that PI3K regulates S6K1 activation via Akt. In some cells, there is evidence supporting a role of Akt in the regulation of mTOR activation (45, 46), but, in other cells, PI3K/Akt has been reported to regulate S6K1 activation independently from mTOR (47, 48). In limited studies, we found that infection of the dominant negative MEK, and MEK inhibitors U0126 and PD98059, block 5-HT–induced phosphorylation of S6K1 but not that of Akt. Thus, there appears to be an additional pathway to that of Akt, through which S6K1 is activated in these SMCs.

Another finding in our study is the involvement of ROS in regulation of Akt phosphorylation. We found that a variety of antioxidants, and the NADPH oxidase inhibitor diphenyleneiodonium, prevent 5-HT–induced Akt phosphorylation. We have previously demonstrated that the mitogenic effect of 5-HT on

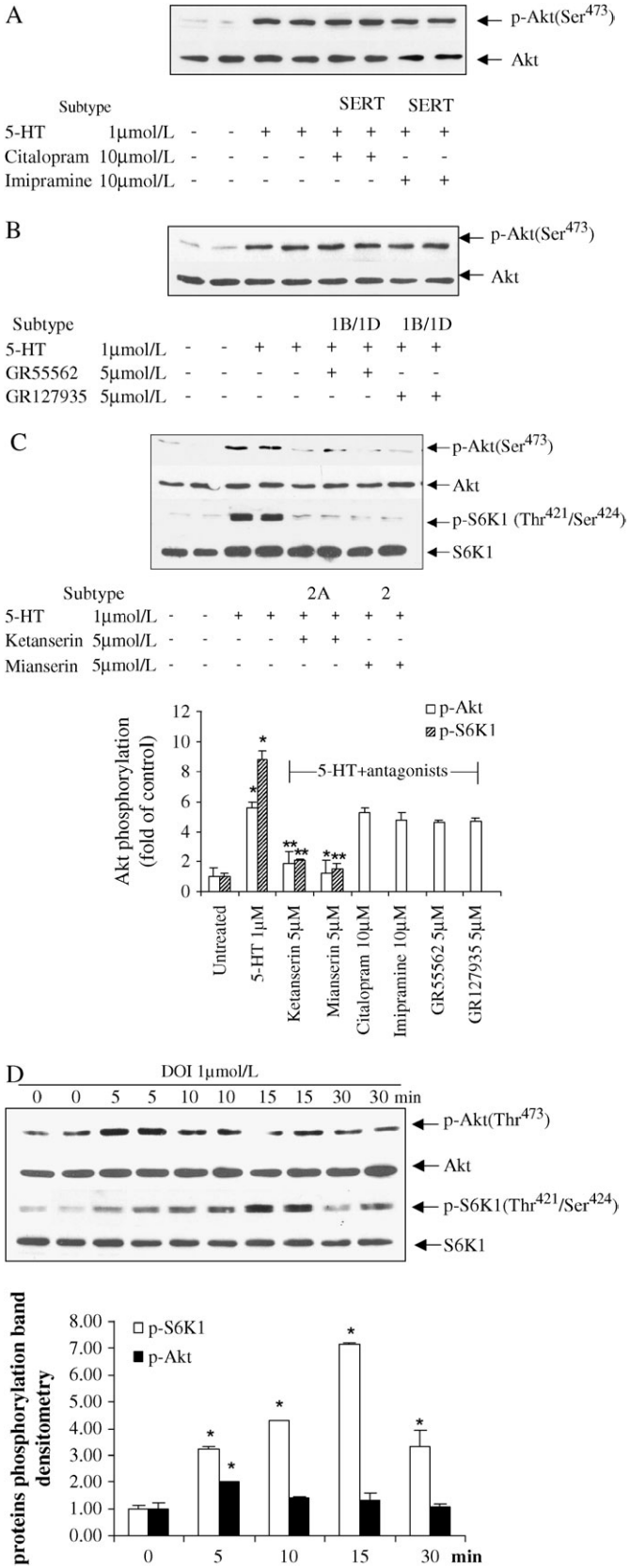

Figure 7. 5-HT 2A receptors mediate Akt and S6K1 phosphorylation. Quiescent SMCs were pretreated with serotonin transporter (SERT) inhibitors citalopram and imipramine (*A*), 5-HT 1B/1D receptor antagonists GR55562 and GR127935 (*B*), 5-HT 2A receptor antagonist ketanserin, or 5-HT 2 receptor antagonist mianserin (*C*), for 30 min, then stimulated with 1 μmol/liter 5-HT for 5 min. Quiescent SMCs were pretreated with with 5HT2 receptor agonist 1-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) for the indicated time (*D*). The phosphorylation of S6K1 (Thr^{421}/Ser^{424}) and Akt (Ser^{473}) were detected by Western blot analysis using phospho-specific antibodies. Total S6K1 and Akt were detected on the same membrane using the total Akt and S6K1 antibodies. Data presented are means ± SD for $n = 4$. * Significant difference from untreated cells at $P < 0.05$; ** significant difference from 5-HT treatment alone at $P < 0.05$.

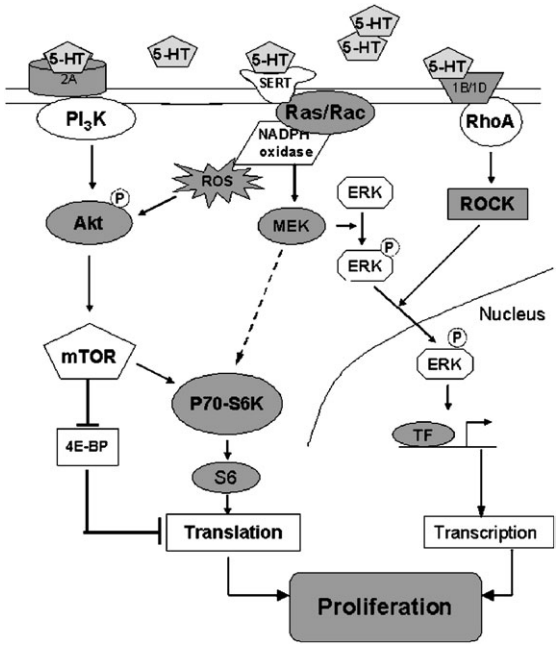

Figure 8. Diagram of signaling pathways for 5-HT–induced SMC proliferation.

PASMCs requires activation of a NADPH oxidase that produces O_2^-, and ERK MAPK activation by 5-HT is dependent upon the generation of ROS (1, 2). Some studies have suggested that PI3K/Akt is a redox-sensitive pathway (49–52). In some cell types, exogenous H_2O_2 has been shown to be capable of activating PI3K and Akt phosphorylation (49, 53, 54). A recent report demonstrated a redox-sensitive Cys^{118} on the small GTPase Ras (55). H_2O_2 from NADPH oxidase modified Ras at Cys^{118} and increased its activity, leading to Akt phosphorylation. In addition, the PI3K suppressor, PTEN, was also found to be a redox target in mediating oxidant-activated PI3K/Akt signaling (56, 57). The molecular target of ROS in mediating PI3K/Akt and ERK activation by 5-HT is in need of further identification.

We have previously demonstrated that SERT is involved in mitogenesis of SMCs caused by 5-HT (2, 20). Additionally, we have recently found that a 5-HT 1B/1D receptor participates in the mitogenic response, and that this receptor is involved in activation of the Rho/ROCK response that translocates ERK MAPK from the cytoplasm to the cellular nucleus (3). We concluded from this observation that there is a combinatorial action of SERT and 5-HT receptor(s) in the mitogenic response of SMCs to 5-HT. Our present data take this observation one step further. From our current data, activation of the Akt/S6K1 pathway by 5-HT appears to involve a 5-HT 2 receptor, and not the 5-HT 1B/1D receptor. Participation of the Akt/S6K1 pathways is necessary for the mitogenic response. These observations support a thesis that there may be a complex structure of SERT and 5-HT receptors that respond to 5-HT, resulting in SMC proliferation. Such a hypothesis would be consistent with the previously reported findings of a block in hypoxia-induced pulmonary hypertension in experimental animals by both knockout of SERT (24, 25) and the use of pharmacologic inhibitors of 5-HT receptors (39, 49, 58, 59). It would also be consistent with a recognition of functional interactions between receptors and SERT in 5-HT–induced SMC contraction (60).

Conflict of Interest Statement: Neither author has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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